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We have utilized a xenograft model of early human breast cancer progression to identify genetic and cellular changes that occur during breast cancer development and to experimentally manipulate these changes to determine which alterations play a causal role in progression. Alterations in relevant markers, viz., ER/PgR, p53, DNA methylation status of ER gene and genomic instability were examined to determine if these correlate with progression. Our data show that conformation altered wild type p53 (CAp53) is overexpressed in MCF10AT system cells which is defective in both DNA binding activity and in its ability to function as a transcription factor. We also show that the activated ER gene in MCF10AT system cells is functional based on its ability to mediate E_2-regulated increase in transcription from both endogenous and exogenous genes. Transcriptional activation of the endogenous ER gene does not appear to be related to a change in methylation status of the gene. E_2 significantly enhanced both the number and size of soft-agar colonies formed by MCF10AT3c cells, a line from a third generation of MCF10AT xenograft lesion. This suggests that xenograft passage has selected for growth regulatory pathways that are E_2-responsive and that identification of these pathways and their role in progression will aid in determining how E_2 acts to increase risk of breast cancer.

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FOREWORD

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INTRODUCTION

Breast cancer is the most common malignancy among women in North America. Although very little is known about the molecular events underlying the development of mammary tumors in humans, much evidence indicates that the presence of estrogen receptor (ER) in mammary carcinoma is generally a good prognostic marker because ER^+ tumor cells are usually more differentiated and have lower metastatic potential. It is generally thought that hormones, including estradiol (E_2), act as tumor promoters by virtue of their effects on cell growth and differentiation, efforts of many investigators have focused on the mitogenic effects of E_2, or E_2 combined with progesterone. Only 6-10% of normal human breast epithelial cells are ER^+, yet more than 60% of primary human breast tumors are ER^+ and initially depend on E_2 for growth (2-4). This would suggest that: (1) ER^+ cells are more likely to become transformed than ER^- cells and/or that the hormonal and cellular milieu of the breast is more conducive to growth of transformed ER^+ cells or (2) if ER^+ expression provides a growth advantage, the cells must undergo some additional change that allows them to avoid the growth inhibitory or differentiating effects of E_2. This would predict that in at least some cases, initially slow growing ER^- tumors could give rise to ER^+ tumors with an increased potential for growth and progression (5).

In the human breast, a spectrum of microscopic changes has been termed proliferative breast disease (PBD). Although hyperplastic lesions are observed in human breast, their role in disease progression is not understood. The progression of histopathological features of PBD has been correlated with increased risk for the development of invasive carcinoma.

The aim of the proposed study is to determine (a) the mechanism by which E_2 influences neoplastic progression of ER^+ and ER^- human breast epithelial cells, and (b) whether stage-specific epigenetic and genetic changes occur during progression of precancerous human breast epithelial cells. We have utilized a xenograft model of early human breast cancer progression to address these questions. This model, a Ha-ras-transformed derivative of MCF10A (ER^-, PR^-) referred to as "MCF10AT", is a line of preneoplastic human breast epithelial cells which are able to grow in immunedeficient mice where they undergo a sequence of progressive histological changes culminating in cases of frank neoplasia in about 25% of the animals (6). Immortalized human breast epithelial MCF10A cells (7) are unable to survive long term in immune deficient nude/beige mice. Thus, MCF10AT and derivatives obtained from successive transplant generations (MCF10AT.TGn, viz., MCF10AT1, MCF10AT2b, MCF10AT3b, etc.) provide a transplantable xenograft model of human PBD with proven neoplastic potential. This model, collectively referred as the "MC10AT" system affords the opportunity to identify genetic and cellular changes that occur during early breast cancer development and to experimentally manipulate these changes to determine which alterations play a causal role in progression.

We have previously demonstrated that endogenous ER expression is activated only in MCF10A cells that express mutant Ha-ras, i.e., the MCF10AT system. We have also shown that the activated ER protein is functional based on its ability to mediate E_2-regulated increase of transcription from a transfected E_2 regulated gene, ERE-TKCAT. These observations further

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strengthened the utility of this model for studying the role of estrogen in early breast cancer development.

BODY OF THE REPORT

Cell Culture:

MCF10A and MCF10A derivatives were maintained in phenol red-free DMEM/F-12 medium supplemented with 5% equine serum, 0.1 µg/ml cholera toxin, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 0.02 µg/ml epidermal growth factor, 100 i.u./ml penicillin and 100 µg/ml streptomycin. Charcoal-stripped serum was not used since it affects the proliferative ability and/or viability of MCF10A cells possibly due to removal of essential growth factors. Only serum that was not able to support growth of the ER^+ cell line, MCF-7, (an indication that it was free of biologically significant amounts of E_2 or other estrogenic compounds) was routinely used.

Determination of endogenous ER function

1) Analysis of progesterone receptor (PgR) expression.
Method: Cell lysates prepared from MCF10AT and MCF10AT3c cells cultured in phenol red-free DMEM/F12-supplemented medium with or without added E_2 (10^{-9} M) were incubated with anti-human PgR mouse monoclonal antibody (Oncogene Science, Cambridge, MA) overnight at 4°C. Immune complexes were pelleted with Protein G Sepharose, solubilized by boiling in SDS-sample buffer and subjected to SDS-PAGE and Western blot analysis. Lysates prepared from the PgR-positive cell line, T47D and treated in the same way were used as controls. Proteins transferred to Immobilon P (Millipore Corp., Bedford, MA) membranes were incubated sequentially with the same anti-PgR antibody and ^{125}I-labeled goat anti-mouse IgG (ICN Radiochemicals, Irvine, CA).
Results: The majority of PgR protein detected in lysates from positive control T47D cells had an electrophoretic mobility corresponding to that of the A (Mr ~83 kDa) and B (Mr ~112 kDa) forms of PgR with a trace of protein with an apparent Mr ~52 kDa (Fig. 1, lane 1). In contrast, the bulk of protein binding anti-PgR antibody from lysates of E_2-treated MCF10AT and MCF10AT3c cells had the mobility of a 52 kDa protein and contained only trace amounts of PgR protein with the mobility of A form PgR and no detectable B form (Fig. 1, lanes 2 and 3). The PgR-A protein in MCF10AT3c cells migrated slightly slower than those seen in the positive control breast cancer cell line T47D and MCF10AT cells. This difference in mobility may be due to the difference in protein loading or may reflect a difference in the level or number of sites of phosphorylation. Alternatively, the difference in migration may be due to the presence of isoforms that may not arise from multiple phosphorylation events (8). Samples prepared from untreated MCF10AT and MCF10AT3c cells had barely detectable levels of the 52 kDa protein and none of the larger forms (Fig. 1, lanes 4 and 5). As the PgR epitope recognized by the monoclonal antibody used in these experiments has not been localized, the origin of the E_2 inducible 52 kDa protein remains to be established. One possibility is that it is a recently reported 45-50 kDa C form of PgR (8). Translation from an internal initiation codon AUG encoded in exon 2 (Met^{595}) has been shown to lead to synthesis of a 45-50 kDa PgR termed the C receptor (8). The

supposition that the 52 kDa protein we have observed is, or is similar to, the C form of the PgR is supported by the observation that anti-PgR antibodies that recognize epitopes in the amino-terminal domain encoded in exon 1 do not detect PgR in MCF10AT and MCF10AT3c (data not shown). Ongoing molecular and functional characterization of the 52 kDa protein should determine the relationship between the 52 kDa protein and the 83 kDa PgR and clarify the potential role of the 52 kDa protein in progression in the MCF10AT system. Our results clearly demonstrate that synthesis of both the small amount of 83 kDa PgR and the 52 kDa protein is induced by E_2 in an ER-mediated process.

2) Effect of E_2 on anchorage independent growth of MCF10AT3c cells

Method: 4×10^4 MCF10AT or MCF10AT3c cells were seeded in 2 ml of 0.3% agar in supplemented DMEM/F12 that was phenol red-free. This suspension was layered over 1 ml of 0.9% agar medium base layer in 35-mm dishes (Costar) and overlayered with 2 ml of phenol red-free DMEM/F12 containing $10^{-8}M$ or $10^{-9}M$ E_2 or an equivalent concentration of vehicle (ethanol). All dishes were incubated at 37°C in 5% CO_2:95% O_2 for 4 weeks with twice-weekly media changes. All cultures were examined 24 h after plating and cell aggregates that might bias final results were marked. Plates with more than 10 aggregates were discarded. Colony forming efficiency (CFE) was calculated by dividing the number of colonies larger than 50 μm (sized using a calibrated ocular grid) by the number of cells seeded. Ten microscopic fields were counted to calculate the total number of colonies/well for the whole well; reported values are the average count from triplicate wells. The number of colonies in different size ranges [50-100 μm, 100-200 μm, 200-300 μm and >300 μm] was calculated in the same manner.

Results: We have not been able to reproducibly demonstrate the effect of E_2 on monolayer proliferation of MCF10AT system cells. However, MCF10AT system cells have enhanced capacities for anchorage independent growth, whereas MCF10A cells do not form colonies in soft agar. Under the conditions of assay utilized in our experiments, MCF10AT cells had a colony forming efficiency (CFE) of 10% with the majority of the colonies smaller than 100 μm in diameter. E_2 had no significant effect on either the CFE or the average colony size of MCF10AT cells (data not shown). However, E_2 treatment did lead to the rare appearance of colonies with diameters >200 μm (~1/8 x10^4 colonies). In the absence of E_2, MCF10AT3c cells had a CFE of 25% and approximately 40% of these colonies had a diameter >100 μm; none had a diameter >200 μm. The CFE of MCF10AT3c cells showed a dramatic increase when the cells were exposed to physiological concentrations of E_2 (Fig. 2). E_2 treatment also enhanced the rate of growth in soft agar with ~ 1/40-80 colonies larger than 200 μm.

Determination of mechanism of ER activation.

Methylation status of ER. The ER gene contains several CpG islands in its promoter and first exon that are rich in sites recognized by methylation sensitive restriction endonucleases. There are several reports indicating that methylation of a number of these sites is correlated with silencing of ER (9,10). A map of CG sites in the CpG island at nucleotides 650-900 (from GCG data bank sequence of human ER gene -M12674) and sequence of CpG island immediately surrounding the NotI site in the first exon of the human ER gene is shown in Fig. 3A.

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Method: The % methylation at each site was determined by PCR sequencing of a minimum of 7 individual clones of bisulphite deaminated genomic DNA from MCF-7 and MCF10A cells. Results: Our results show a loss of methylation at most of these sites in MCF10A cells (Fig. 3A). Methylation of genomic clones from MCF10AT and transplant generations showed some variations in methylation pattern from MCF10A cells but maintained the same overall distribution seen in the parental line (Fig. 3A).

2) The methylation status of the CpG island in exon 1 was also examined by Southern blot analysis of genomic DNA digested with the methylation sensitive enzyme, NotI, and the methylation insensitive enzyme, EcoRI. The 3.1 kbp EcoRI fragment containing this CpG island has a single NotI site. NotI cleaves the 3.1 kbp fragment, releasing 1.2 and 1.9 kbp fragments if the site is completely unmethylated (11).

Method: Ten μg of purified genomic DNA was digested with EcoRI (4 units/μg DNA) and NotI (4 units/μg DNA) for 16 h. NotI will only cleave DNA at its recognition site (GC/GGCCGC) if the indicated C residue in this site is unmethylated on both strands (12). This means that methylation by mammalian C-5- DNA MTase at either CpG dinucleotide in the NotI site will prevent cleavage. The resulting DNA fragments were separated by electrophoresis on 2.5% agarose gel, blotted and probed with a 0.3 kb EcoRI/PvuII fragment of the ER gene obtained from the plasmid POR3 (ATCC-57681). Completeness of cutting was verified by determining that cleavage was complete at a c-myc CpG island NotI site that is normally unmethylated in vivo (11).

Results: Our results (Fig. 3B) indicate that this NotI site is unmethylated in a majority of MCF10A cells and that the proportion of methylated to unmethylated NotI sites in exon1 does not change in MCF10AT and its derivatives. In contrast, this NotI site is fully unmethylated in MCF-7 cells (Fig. 3B).This suggests that even though loss of methylation at this NotI site may be an indicator of a generalized lack of methylation in the CpG islands of the ER and of active ER transcription in breast tumor cells and lines (9,10), loss of methylation at the NotI site in exon1 is not sufficient to allow activation of ER transcription in MCF10A cells.The low level of methylation at this NotI site is similar in both ER⁻ MCF10A cells and ER⁺ MCF10AT and MCF10AT3c cells. (Fig. 3B). These results do not rule out the possibility that methylation of other C residues in the exon 1 region or at upstream sites in the ER promoter plays a role in regulating ER expression. We are currently evaluating this by analyzing methylation of all C residues in the promoter and exon 1 regions of MCF10A and MCF10AT ER genes.

Evaluation of genomic instability.
In order to determine to what extent introduction of T24 Ha-ras increases the genomic instability of parental MCF10A cells, and whether further increases in genomic instability occur in MCF10AT cells during passage through successive transplant generations (TG1, TG2, TG3, TGn), an indirect measure of genomic instability is being used. The capacity for development of resistance to N-(phosphonoacetyl)-L-aspartate (PALA) depends on the ability of a cell to amplify the gene coding for the multifunctional protein containing the enzyme activities, carbamoyl synthase, aspartate transcarbamylase and dihydroorotase (CAD). The method developed by Tlsty (13) has been successfully used to determine differences in genomic stability of cells from a Syrian

hamster model of neoplastic progression (14). Plating efficiency and cell cycle times and LD$_{50}$ for all cells was determined in phenol red-free growth medium.

Method: Cells were seeded in appropriate numbers to give 2 x 10^5 to 2 x 10^6 cells/150 mm culture dish and selected for ability to form colonies at a stringency of 9 x the LD$_{50}$ of PALA. A minimum of 2 x 10^8 viable cells were examined for each population. A representative selection of at least 6 colonies were subcloned and reexposed to 9 x LD$_{50}$ to assure true drug resistance.

Results: The frequency of PALA resistant colony formation (FPRC) for MCF10A and MCF10AT system cells at 3x, 6x and 9x LD$_{50}$ is shown in Table 1. MCF10AT cells exhibited ~40-fold increase in frequency of FPRC compared to MCF10A cells (Fig. 4 and Table 1) wheras cell lines derived from second to fifth transplant generations (MCF10AT2b, MCF10AT3b, MCF10AT4d and MCF10AT4j) showed a marked decrease in FPRC (10^{-5} to 10^{-7}) when compared to their parental line, MCF10AT (Fig. 4 and Table 1). Our data show that introduction of T24 Ha-ras into MCF10A cells converts these cells to a state that is permissive for development of PALA resistance at higher frequencies. However, there is no correlation between frequency of resistance to PALA and phenotype of lesion from which the various MCF10AT transplant generations were derived.One of the reasons for this may be that during successive transplantation in mice, a selection against cells that give rise to PALA-resistant variants may have occurred in vitro during subsequent tissue culture.

Analysis of p53 protein expression

The importance of mutations in the coding regions of the p53 gene to development of breast cancer is underscored by the identification of such mutations in 60-70% of human breast carcinomas tested. A number of human breast cancer cell lines have been shown to express only the mutated form of p53. Deletions of 17p (which may include the *TP53* locus) with loss of heterozygosity have been noted in ~60% of breast tumors. Monoclonal antibodies prepared against several regions of the p53 protein have been used to identify overexpression and nuclear localization of the protein in both in situ and invasive carcinoma of the breast. These studies suggest that p53 mutations may be an early and consistent event in breast carcinoma induction.

Previously we have shown an increase in the levels of total p53 protein expressed in MCF10AT system cells. We have also shown that this increase in p53 protein levels corresponded with a rapidly migrating form of P53 that was detected with mAb pAb421(recognizes a motif in the carboxyl terminus of P53). Immunoprecipitation experiments with the mAb pAb240 (recognizes the amino acid motif RHSVV, residues 213-217 in human P53 which is cryptic in wild type P53 but commonly exposed in many oncogenic mutants) yielded a pattern that corresponded with the rapidly migrating forms detected with PAb421. These data indicated that the PAb240-reactive form of P53 could either be mutant or a conformationally altered form of wild type p53. SSCP and DNA sequence analysis showed that p53 mRNA expressed in MCF10AT system cells is normal. Taken together, these data indicate that the majority of wild type p53 protein expressed in MCF10AT system cells exist in a conformationally altered state rather than in native conformation.

Immunocytochemical detection of P53:

Method: Cells were plated on 8-well chamber slides at approximately 2×10^4 (MCF10A and MCF10AT system cells) to 4×10^4 (MCF-7) cells/well. After 2-3 days, the slides were rinsed twice with PBS and fixed in methanol:acetone (1:1) for 3 min at -20°C. The slides were dried at room temperature for 20 min and washed with PBS. Endogenous peroxidase was inactivated with 0.3% H_2O_2 and non-specific binding blocked with 1% BSA. Cells were incubated with pAb1801 or pAb240, Oncogene Science, following manufacturer's directions. The bound Ab was visualized using an ABC kit from Vector Labs. HepG2P9T2 cells which express high levels of SV40T antigen and accumulate high levels of nuclear P53 were used as a positive control for P53 staining. MCF-7 cells were included as an additional control for staining of breast cells that produce only normal P53. Negative controls were incubated with 2 μg/ml normal mouse IgG substituted for pAb1801 and pAb240. All slides were lightly counterstained with hemotoxylin.

Results: The distribution of P53 in nuclei and cytoplasm of MCF10A and MCF10AT system cells detected by using pAb1801 and 240 is shown in Table 2. Using either pAb240 or pAb1801(an antibody directed to NH_2 terminus of P53 and recognizes both native and conformationally altered P53), the percentage of nuclei exhibiting uniform staining was similar in MCF10A and MCF10AT system cells with the exception of MCF10AT3b. On the other hand, a significant enhancement in the staining of stippled nuclei and cytoplasm was observed in all MCF10AT derivatives with the mAb pAb240 (Table 2). The pattern of staining of MCF-7 cells included as a control for P53 staining of breast cells was identical to that reported by Bartek et al (15).

Evaluation of Functionality of P53 in the MCF10AT system. The functional status of P53 was assessed by testing its ability to bind to p53-response elements and to activate transcription from a p53-responsive promoter, such as WAF1.

1) Analysis of DNA binding activity: The growth suppressor function of wild type p53 in vivo is believed to require sequence-specific binding to DNA. Wild type p53, but not mutant p53, binds in vitro to consensus sequences matching the consensus 5'-(Pu)$_3$ C(A/T) (A/T)G(Py)$_3$-3' (16).Whole cell extracts prepared from MCF10A, MCF10AT, MCF10AT1, MCF10AT2b, MCF10AT3b and MCF10AT3c were tested for DNA-binding activity by electrophoretic mobility shift assay. We used the natural p53-binding site from the WAF-1 gene, which differs from the consensus at 2 nucleotide positions as double stranded oligonucleotide probe.

Method:

Preparation of cell extracts. MCF10A and MCF10AT system cells were plated on 100-mm dishes and grown to 75% confluency at 37°C as described above. Whole cell extracts were prepared by three cycles of freezing and thawing in 0.1 ml of buffer containing 20 mM Hepes (pH 7.9), 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.4 M KCl, 1 mM PMSF, 1 μg/ml each of leupeptin, aprotinin, and pepstatin, and 20% glycerol. Extracts were dialyzed, clarified by centrifugation and stored at -70°C. Protein concentrations were determined by Bradford method.

DNA binding Assay. 15 μg of protein were preincubated with 2 μg poly(dI-dC) in the presence or absence of 0.5 μg mAb pAb421 or pAb1801, or both for 30 min at room temperature in 20 μl of binding buffer (20 mM Hepes, pH 7.9, 1 mM DTT, 50 mM KCl, 5 mM $MgCl_2$, 10 μM $ZnSO_4$, 0.1 mg/ml BSA, 0.1% NP40, and 5% glycerol). 5 ng of ^{32}P-labeled ds WAF1 p53 binding site

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(top strand, 5'-aagtggatcc-GAACATGTCCCAACATGTTg-3'; bottom strand, 5'-aggaagatct-cAACATGTTGGGACATGTTC-3') was added and incubated for another 30 min at room temperature. Competitor oligonucleotides were added during the preincubation step. The nonspecific oligonucleotide competitor contains a vitamin D responsive element from the human osteocalcin gene (top strand, 5'-aagtggatcc-TTGGTGACTCACCGGGTGAACGGGGGCATTG-3'; bottom strand, 5'-aggaagatct-CAATGCCCCCGTTCACCCGG-TGAGTC-3'). Oligonucleotides were annealed and 5' overhangs were filled in with the Klenow fragment of E.coli DNA polymerase I and dATP, dGTP, dTTP and either dCTP (competitors) or α-[^{32}P]dCTP. Reaction mixtures were analyzed by electrophoresis at 160 V on 6% polyacrylamide gels (80:1, acrylamide:bisacrylamide) in 0.5X Tris-Borate containing 1 mM EDTA under cooling by water circulation (8-10°C). Following electrophoresis, gels were dried and autoradiographed. Results: When MCF10A extracts were incubated with the probe, a faint but specific P53-DNA complex formation was observed (Fig. 5). The addition of pAb421 significantly enhanced the formation of P53-DNA complex that could be seen as a retarded band (Fig. 5). Addition of pAb1801 which binds to the NH2-terminal region of P53 does not enhance complex formation as pAb421 (Fig. 5). The presence of P53 in the pAb421-enhanced P53-DNA complexes was further confirmed by supershifting the band with pAb1801 (Fig. 5). These results demonstrate that both p53 and pAb421/pAb1801 are present in this protein-DNA complex. The specificity of P53-DNA complexes was examined by competition experiments (Fig. 5). Only oligonucleotides containing the p53 binding sites competed effectively in the formation of this retarded band whereas the nonspecific oligonucleotide competitor containing the vitamin D responsive element did not affect complex formation (Fig. 5). Similar DNA binding results were obtained with MCF10AT cell extracts. Unlike MCF10A and MCF10AT cells, MCF10AT derivatives do not have the ability to complex with WAF-1 DNA (Fig. 6). However, DNA complex formation ability is specifically restored in the presence of pAb421 and not by pAb1801 (Fig. 6). This difference in DNA binding activity in MCF10AT derivatives may be attributed to the predominant existence of wild type P53 in a conformationally altered state that is unable to bind to consensus DNA unless its conformation is specifically restored by allosteric mechanisms that involve the carboxyl terminus of p53 which is triggered by the binding of the carboxyl-terminal antibody pAb421. Similar treatment with pAb421 of wild type p53 protein expressed in baculovirus, bacterial or in vitro translation systems has been shown to be necessary for activation of DNA binding (17).

2) Analysis of Transcription Activation Function of P53

The functionality of p53 with regard to its ability to function as a transcription factor was examined. For this, MCF10A, MCF10AT, MCF10AT2b, and MCF10AT3b cells were transfected with pWAF1/βgal alone or together with the wild type p53 expression vector pC53SN3.
Method:
Plasmid Constructs: The wild-type p53 expression vector (pC53-SN3) was a gift from Dr. B. Vogelstein. In this plasmid, p53 is expressed from the CMV immediate early promoter. The plasmid, pCMV-neo is identical to pC53-SN3 except that it lacks p53. The WAF1 promoter-βgal reporter plasmid, pWAF1/βgal, was a gift from Dr. P. Abarzua.
Transfection: MCF10A, MCF10AT, MCF10AT2b, or MCF10AT3b cells (5 x 10^5 cells per 60 mm dish) were plated 18 h prior to transfection in media as described above. The cells were

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transfected using the calcium phosphate procedure. To determine the functionality of endogenous P53, cells were transfected with varying amounts of pWAF1/βgal (2.5 - 10 μg). The ability of MCF10A and MCF10AT system cells to support transcription activation mediated by exogenous wild type P53 was determined by cotransfecting cells with 5 μg of pWAF1/βgal and varying amounts of pC53-SN3 (0.5, 1, 2.5 and 5 μg). 5 μg of pWAF1/βgal was chosen as cells transfected with this amount yielded maximal reporter activity that was in the linear range. In both sets of transfection experiments, the empty pCMVneo was used as carrier DNA to adjust the final concentration of DNA to 10 μg. Cells were incubated with plasmid DNA for 4 h, followed by 3 min incubation with 15% glycerol. Cells were lysed 30 h following transfection and βgal activity assayed by ELISA.

Results: As shown in Fig. 7, a significant decrease in the ability of endogenous P53 to transactivate β-gal reporter from WAF-1 promoter was observed in MCF10AT system cells as compared to MCF10A cells, with lowest levels of β-galactosidase activity in MCF10AT3b cells. The differences in reporter activity observed in MCF10AT system cells is not related to variations in transfection efficiencies or cell viability as cells transfected independently with pCH110 yielded comparable levels of β-gal activity. These data suggest that although the MCF10AT system cells express higher levels of wild type P53 than MCF10A cells, its ability to function as a transcription factor is significantly impaired probably due to its presence in the conformationally altered state.

In order to test whether high levels of native wild type P53 attained by introduction of exogenous wild type p53 could stimulate reporter activity beyond that achieved by endogenous P53, MCF10A and MCF10AT system cells were cotransfected with pWAF1/βgal and various amount of pC53SN3. Our results showed that introduction of exogenous wild type p53 caused a dramatic drop in β-gal activity in all the cell lines tested. However, the patterns of reporter stimulation observed in the presence and absence of pC53SN3 were the same (Fig. 7), i.e., MCF10A cells showed highest activity and MCF10AT3b showed the lowest. Maximal levels of reporter activity was obtained with lowest dose of pC53SN3 whereas levels greater than 2.5-5 μg drastically reduced the viability of the cells. This is not unexpected as p53 expression from pC53SN3 is under the control of CMV, a strong viral promoter. These data indicate that in MCF10A and MCF10AT system cells wild type P53 is not limiting since introduction of exogenous wild type p53 failed to further enhance β-gal activity from the WAF1 promoter. However, since cells expressing higher levels of conformationally altered P53 show proportionately lower levels of reporter stimulation, these data suggest that conformationally altered P53 is defective in sequence specific DNA binding and in its ability to function as a transcription factor. Our data from cotransfection experiments suggest that conformation altered P53 present in MCF10AT system cells probably influences the functionality of expressed wild type P53 by trapping it in oligomer complexes. Alternately, the cellular milieu of MCF10A cells is conducive to the existence of expressed wild type P53 in the conformation altered state. However, the reactivity of expressed P53 to mAb pAb240 or pAb421 have not been analyzed to test these possibilities.

CONCLUSIONS

Using a xenograft model for progression of human proliferative breast disease, we have show that expression of the endogenous ER occurs only in MCF10A cells that are transfected with T24 Ha-ras. We have demonstrated the functionality of ER based on its ability to mediate estradiol-regulated increase in transcription from both endogenous (PgR) and exogenous (ERE-TKCAT) estrogen-regulated genes. While E_2 had no significant effect on monolayer growth of MCF10AT system cells or on soft-agar colony formation by MCF10AT cells, it significantly increased both the number and size of soft-agar colonies formed by MCF10AT3c cells, a line from a third generation MCF10AT xenograft lesion. This suggests that xenograft passage has selected for growth regulatory pathways that are E_2- responsive and that identification of these pathways and their role in progression will aid in determining how E_2 acts to increase risk of breast cancer.

The mechanism by which T24 Ha-ras is associated with the activation of ER in a ER⁻ line is not clear. There are a number of reports that indicate that silencing of ER expression in breast tumors and breast cell lines is associated with extensive methylation of C residues in the exon 1 CpG island of ER including those in the NotI site examined in the studies reported here. Treatment with 5-azacytidine, which is incorporated into DNA where it acts as an inhibitor of 5-C-DNA methyltransferase, results in partial loss of methylation at a number of CpG sites including the exon 1 NotI site in the ER gene of ER⁻ MDA-MB-231 breast cancer cell line. It also results in expression of functional ER protein, strengthening the suggestion that methylation plays a role in silencing ER transcription. Our data demonstrate 1) that lack of methylation at the NotI restriction site cannot by itself be used as a direct indicator of transcriptional activation of the ER gene in breast cells and 2) that a low level of methylation at the NotI site, which is characteristic of normal breast tissue is not sufficient to allow expression of ER in MCF10A cells. These data do not rule out the possibility that methylation of other C residues in the exon 1 region or upstream sites in the ER promoter plays a role in regulationg ER expression.

The data obtained so far indicate that accumulation of conformationally altered wild type P53 (rather than genetic alterations) that is defective in P53-mediated functions such as transcription activation may play a role in neoplastic progression of the MCF10AT xenograft model.

Goals for year 3.
Work in progress:

1. We are awaiting the approval of a protocol that has been submitted to examine the effect of estradiol on the rate of progression of MCF10AT and MCF10AT1 cells. MCF10AT and MCF10AT1 cells will be grown in estrogen-free media, and 10^7 cells will be injected in the fat pad of 42 mice (21 mice each for MCF10AT and MCF10AT1) of ovariectomized female nude/beige mice. 7 (7/21) mice from each group will also be injected with an estrogen-responsive cell line, viz., MCF-7 cells, to test the efficacy of hormone treatment. 14/21 mice from each group injected with MCF10AT (or MCF10AT1) cells alone or with MCF-7 cells will receive implants of 1.7 mg (6 week release) E_2, and the remaining 7 mice will receive similar implants carrying the vehicle. Experiments will be terminated at 120 days and lesions will be removed from all the animals. Two

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lesions will be selected at random from each test group for recovery of cells. Serial sections from the lesions will be examined immunohistochemically for expression of ER, PgR, p53 and mdm-2. Cells from these lesions will be examined for genetic instability and/or loss of p53 function, c-myc, mdm-2, ER and PgR expression.

2. ER positive clones have been picked from the soft agar assays conducted with MCF10AT3c cells (discussed above). These clones will be analyzed for PgR expression and effect of estradiol on proliferation and progression in nude mice.

3. MCF10AT and MF10AT1 cells have been transfected with exogenous human ER. We are currently in the process of characterizing the clones selected from both transfection experiments. Once characterization is complete these clones will be evaluated for the presence of functional estrogen-responsive pathways as described above.

4. Complete examination of the ER promoter region.

REFERENCES

1.   Russo, J., Gusterson, B.A., Rogers, A.E., et al: Comparative study of human and rat mammary tumorigenesis. Lab Invest. 62:244-278.

2.   McGuire, W., Carbone, P., and Vollmer, R. (eds.): Estrogen receptors in human breast cancer, New York. Raven press, p1995.

3.   Dickson, R., and Lippman, M. (1988): Control of human breast cancer by estrogen, growth factors, and oncogenes. In: M.E. Lippman and R.B. Dickson (eds.), Breast Cancer: Cellular and Molecular Biology, pp. 119-165. Boston, Kluwer Academic Publishers,

4.   Wittligg, J.L. (1984). Steroid hormone receptors in breast cancer. Cancer (Phila.), 53:630-643.

5.   Zajchowski, Deborah, A., Sager, R., and Webster, L. (1993) Estrogen inhibits the growth of estrogen receptor negative, but not estrogen receptor positive, human mammary epithelial cells expressing a recombinant estrogen receptor. Cancer Res. 53:5004-5011.

6.   Miller, F.R., Soule H.D., Tait, L., Pauley R.J., Wolman S.R., Dawson, P.J., Heppner, G.H (1993). Xenograft model of human proliferative breast disease. J. Nat. Cancer Inst. 85:1725-1732.

7.   Soule, H.D., Maloney T.M., Wolman, S.R., Peterson, W.D., Jr. Brenz R., McGrath, C.M., Russo, J., Pauley, R.K., Jones, R.F., and brooks, S.C. (1990). Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF10. Cancer Res. 50:6075-6086.

8.   Wei, L.L., Gonzalez-Aller, C., Wood, W.M., Miller, L.A., and Horowitz, K.B. (1990). 5' heterogeneity in human progesterone receptor transcripts predicts a new amino terminal truncated "C"-receptor and unique A-receptor messages. Mol. Endocrinol., 4:1833-1840.

9.   Lapidus, R.G., Ferguson, A.T., Ottaviano, Y.L., Parl, F.F., Smith, H.S., Weitzman, S.A., Baylin, S.B., Issa, J.P., and Davidson, N.E. (1996). Methylation of estrogen and

progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. Clin. Cancer Res., 2:805-810.

10. Ottaviano, Y.L., Issa, J.-P., Parl, F.F., Smith, H.S., Baylin, S.B., and Davidson, N.E. (1994). Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. Cancer Res. 54:2552-2555.

11. Issa, J.-P., Ottaviano, Y.L., Celano, P., Hamilton, S.R., Davidson, N.E., and Baylin, S.B. (1994). Methylation of the estrogen receptor CpG island links ageing and neoplasia. Nature Genet., 7:536-540.

12. Nelson, M., and McClelland, M. (1989). Effect of site specific methylation on DNA modification methyltransferases and restriction endonucleases. Nucleic Acids Res., 17 Suppl.:389-415.

13. Tlsty, T.D. (1990). Normal diploid human and rodent cells lack a detectable frequency of gene amplification, Proc. Natl. Acad. Sci. USA 90:8891-8895.

14. Jonczyk, P., White, A., Lum, K., Barret, J.C., and Tlsty, T.D. (1993). Amplification potential in preneoplastic and neoplastic syrian hamster embryo fibroblasts transformed by various carcinogens. Cancer Res. 53:3098-3102.

15. Bartek, J., Iggo, R., Gannon, J., and Lane, D.P. (1992). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene, 5:893-899, 1992.

16. El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992) Human genomic sequences define a consensus binding site for p53. Nat. Genet., 1:45-49.

17. Hupp, T.R., Meek, D.W., Midgley, C.A., and Lane, D.P. (1992). Regulation of the specific DNA binding function of p53. Cell, 71:875-886.

Fig.1 Detection of PgR in MCF10AT system cells by immunoprecipitation and Western blot analysis.

Anti-PgR antibody at a concentration of 10 μg/ml was used for immunoprecipitation of PgR from cytosols of E_2-treated and untreated MCF10AT and MCF10AT3c cells, and from T47D cells, a constitutive expressor of PgR. For Western blot analysis of immunoprecipitated samples, 5 μg/ml of the same PgR antibody was used. Lane 1 represents the positive control, T47D cells; lanes 2 and 3 represent MCF10AT and MCF10AT3c cells treated with 10^{-9}M E_2; lanes 4 and 5 represent MCF10AT and MCF10AT3c cells without E_2 treatment.

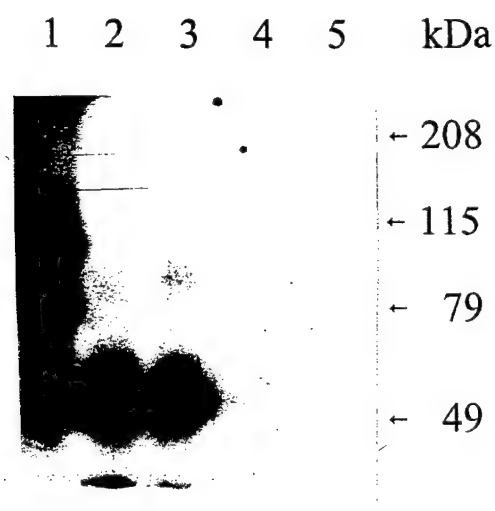| 1	2	3	4	5	kDa
					← 208
					← 115
					← 79
					← 49

Fig. 2. Effect of E_2 on anchorage independent growth of MCF10AT3c cells. Total number of colonies represent colonies greater than 50 μm in diameter. Colonies were counted according to their size: small, medium, large and very large, 50-100 μm; 100-200 μm; 200-300 μm and >300 μm in diameter, respectively. Ten microscopic fields were counted to calculate the number of colonies for the whole well, and average of 3 wells for treated (10^{-9} and 10^{-8}M E_2) or untreated cells was calculated. Results are expressed mean ±SD of 3 experiments.

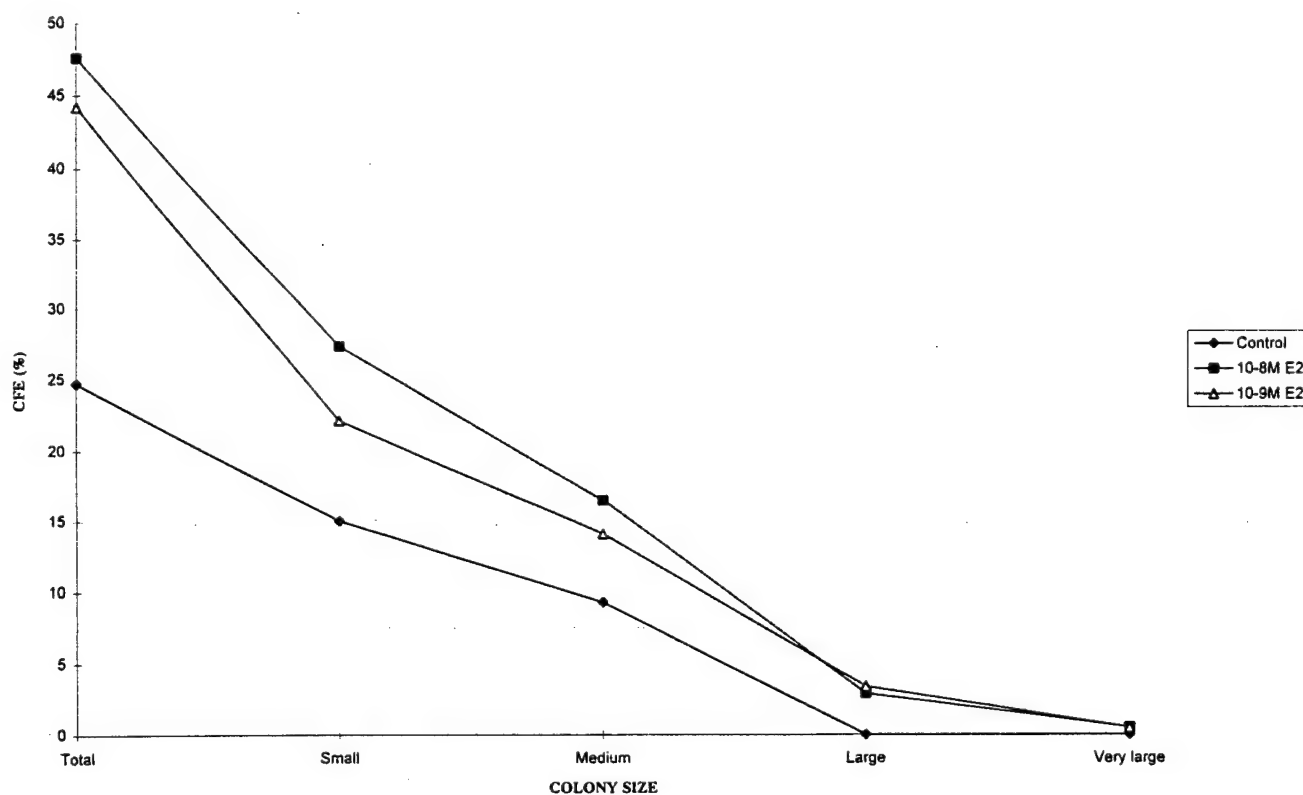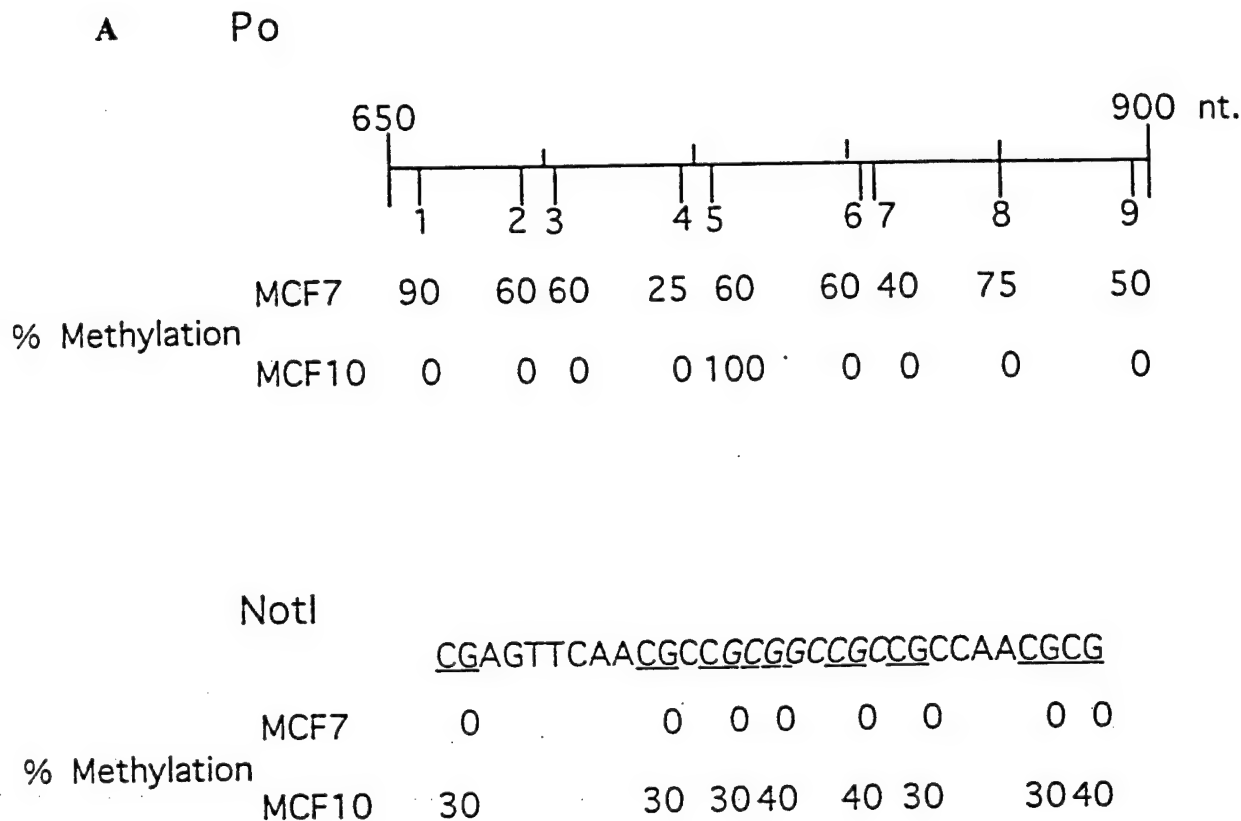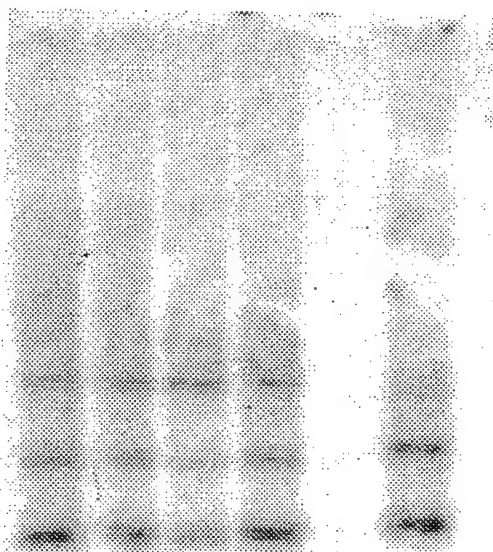Fig. 3. Determination of methylation status of exon 1 of ER gene in MCF10A and MCF10AT system cells.

A Po

650   900 nt.

1	2	3	4	5	6	7	8	9

% Methylation	1	2	3	4	5	6	7	8	9
MCF7	90	60	60	25	60	60	40	75	50
MCF10	0	0	0	0	100	0	0	0	0

NotI

CGAGTTCAACGCCGCGGCCGCCGCCAACGCG

% Methylation								
MCF7	0	0	0	0	0	0	0	0
MCF10	30	30	30	40	40	30	30	40

B   1   2   3   4   5

A. Map of CG sites in the CG island at nucleotides 650-900 (From GCG data bank sequence of human ER gene-M12674) and sequence of CG island immediately surrounding the NotI site in the first exon of the human ER gene. The % methylation at each site was determined by PCR sequencing of a minimum of 7 individual clones of bisulfite deaminated genomic DNA from MCF7 and MCF10A cells. Methylation of genomic clones from MCF10AT and transplant generations showed some variations im methylation pattern from MCF10A cells but maintained the same overall distribution found in the parental line.

B. Southern blot of genomic DNA from MCF10AT3 (Lane 1); MCF10AT (Lane 2); MCF10Aneo (Lane 3); MCF10A (Lane 4) and MCF7 (Lane 5). The blot was probed with a ^{32}P labeled 300 bp fragment of ER cDNA from the exon 1 region. DNA was cut with EcoRI and NotI to give two fragments of 1.2 and 1.9 kbp when the Not I site is unmethylated. Note persistance of the uncut 3.1 kbp EcoRI fragment in samples from MCF10A cells and derivatives indicating that this site is partially methylated in MCF10 cells even after transformation with a mutated ras gene (T24) and several passages through nude mice.

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Fig. 4. Frequency of PALA resistant colony formation in MCF10A and MCF10AT system cells at $9XLD_{50}$ PALA.
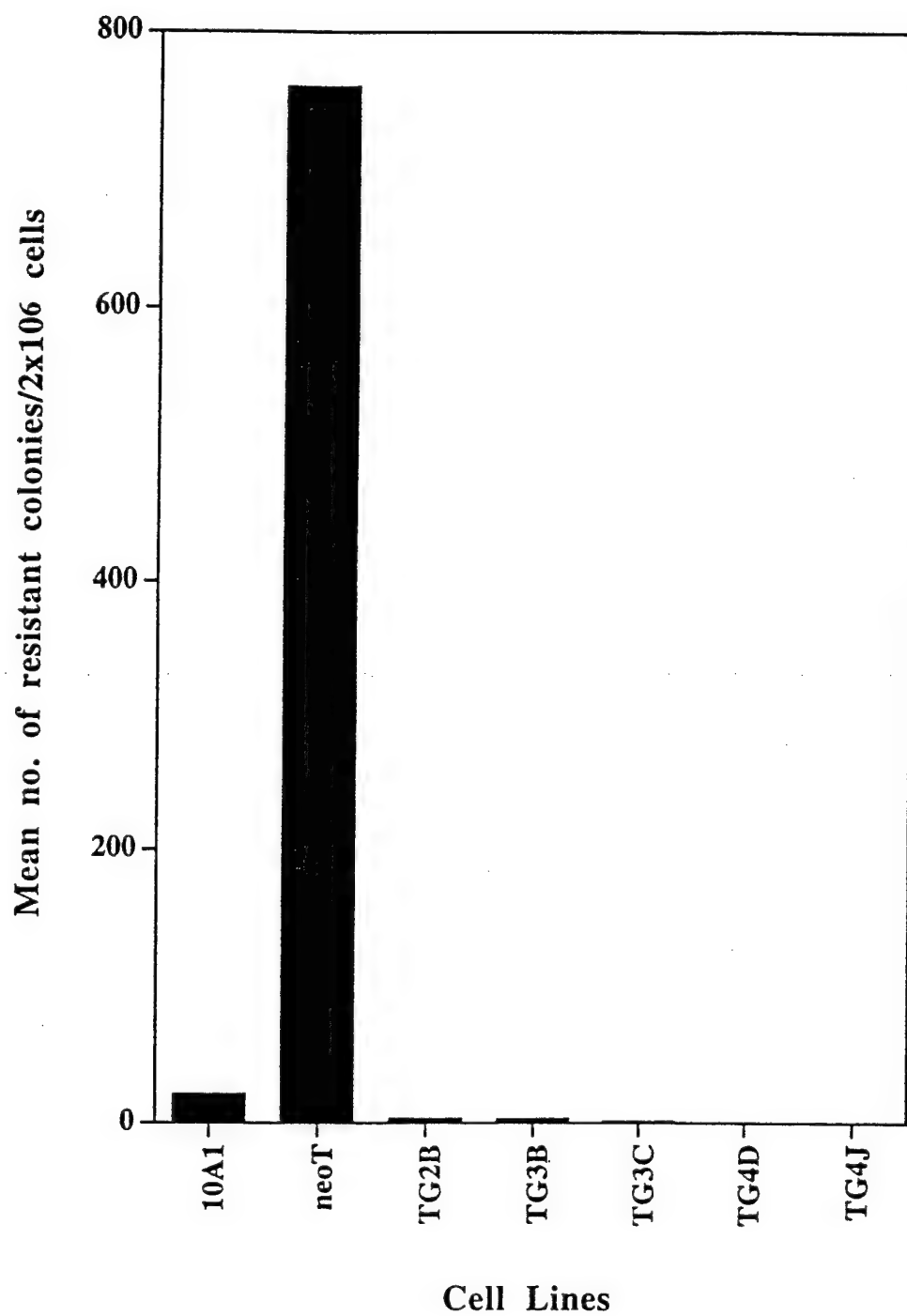
Fig. 5. Analysis of DNA binding activity of P53 from MCF10A cells by electrophoretic mobility shift assay.
Lane 1 represents free WAF1 DNA probe; lane 2, binding in absence of antibodies; lane 3, binding in presence of pAb421; lane 4, binding in presence of pAb421 and 100X unlabeled WAF1 DNA; lane 5, binding in presence of pAb421 and pAb1801; lane 6, binding in presence of pAb1801 and lane 7, binding in presence of pAb1801 and 100X unlabeled nonspecific competitor.
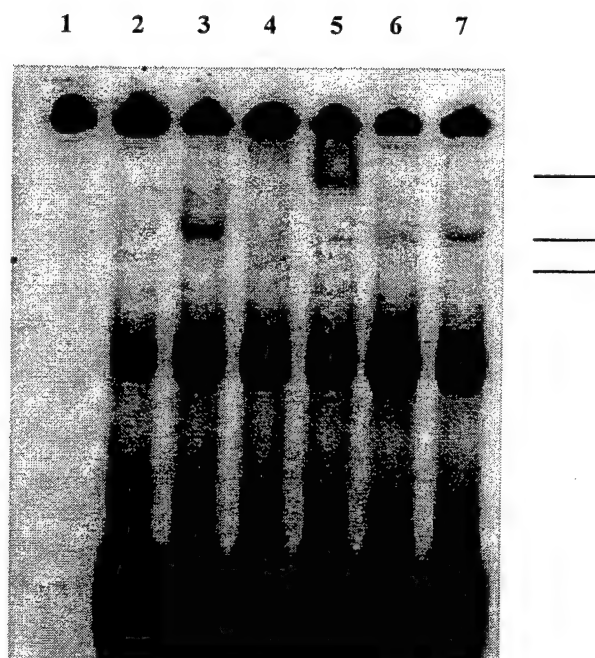
Fig.6. Analysis of DNA binding activity of P53 from MCF10AT system cells by gel mobility shift assay.
Lane 1, binding in presence of pAb421
Lane 2, binding in presence of pAb1801
Lane 3, binding in presence of pAb421 and 1801.
*denotes binding in presence of 100-fold excess unlabeled specific competitor
**denotes binding in presence of 100-fold excess nonspecific competitor.

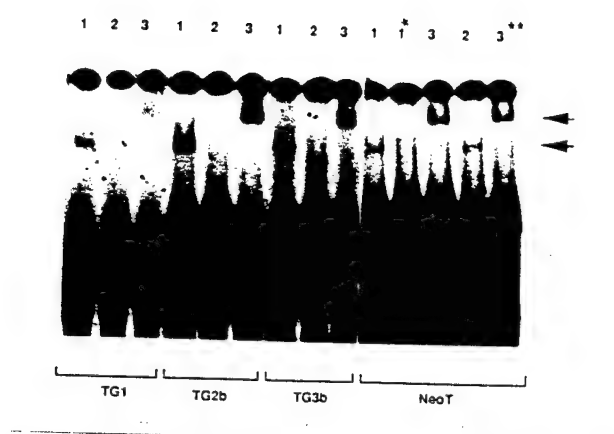Fig.7. Analysis of transcription activation function of P53 in MCF10A and MCF10AT system cells.

Cells were transfected with pWAF1/βgal alone (5 µg) or in combination with pC53-SN3 (0.25-5 µg), an expression vector for wild type p53 under the control of CMV. The total amount of DNA in all samples were maintained at 10 µg with pCMV-neo as carrier DNA. Cell lysates prepared 30 h after transfection were analysed for β-gal (reporter) activity by ELISA. Results obtained from three independent experiments are summarized and graphically represented. Values are expressed mean±SD. Independent transfection of cells with pCH110 yielded comparable levels of β-galactosidase activity confirming that the results were not due to differences in transfection efficiency or cell viability (data not shown).
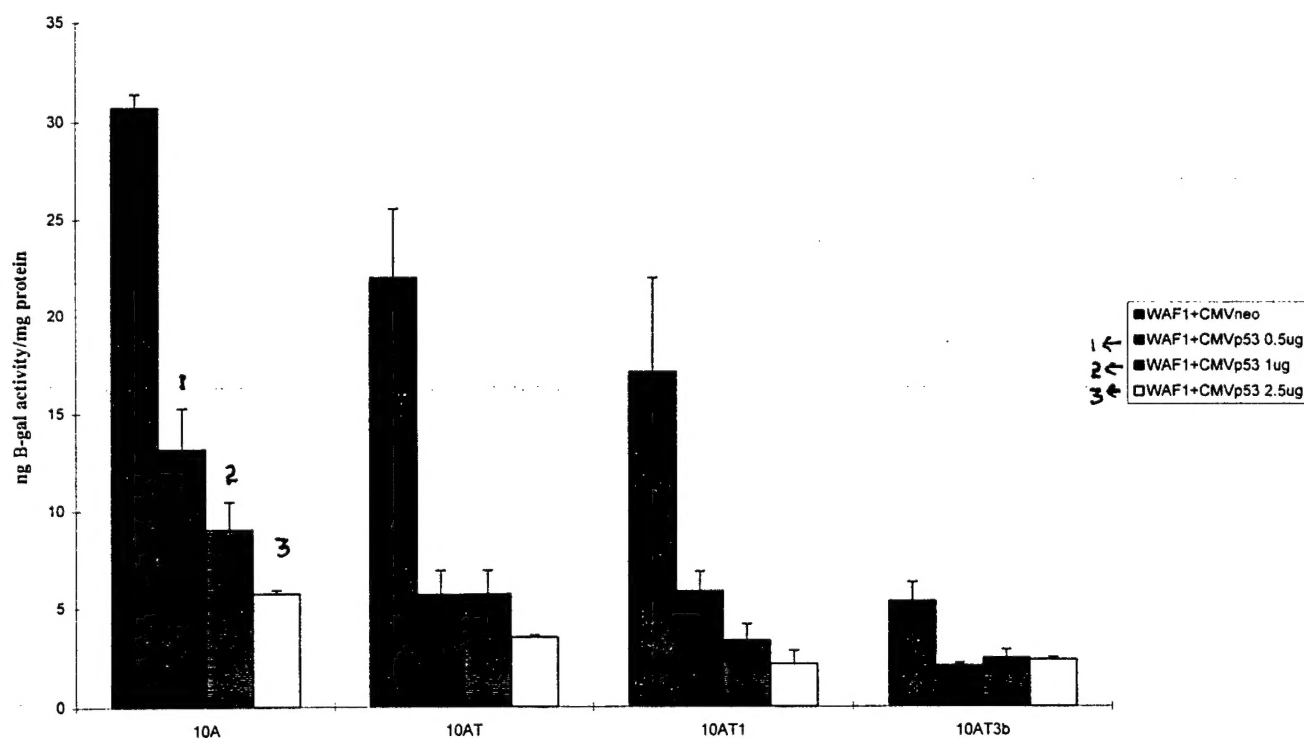
Table 1

Frequency of PALA resistance in MCF10A cell lines

Cell line	Frequency of PALA resistance		
	$3xLD_{50}$	$6xLD_{50}$	$9xLD_{50}$
MCF10A(21-27)	10^{-3}	10^{-7}	$<10^{-8}$
MCF10A1(>150)	10^{-2}	$4x10^{-3}$	10^{-4}
MCF10A1neoT	CM	10^{-2}	$3x10^{-3}$
MCF10A1neoT.TG2B	10^{-3}	$6x10^{-4}$	10^{-5}
MCF10A1neoT.TG3B	10^{-3}	10^{-4}	10^{-5}
MCF10A1neoT.TG3C	$2.2x10^{-4}$	10^{-6}	$<10^{-6}$
MCF10A1neoT.TG4D	$2.7x10^{-4}$	$2.3x10^{-4}$	$<10^{-7}$
MCF10A1neoT.TG4J	$4x10^{-4}$	$1.3x10^{-4}$	$<10^{-7}$

(passage)   CM, confluent monolayer

Table 2

Distribution of P53 in nuclei and cytoplasm of MCF10A, MCF10Aneo, MCF10AneoTcells and MCF10AneoT transplant generations.

CELL LINE	mAb 1801 (normal and conf. altered)		mAb 240 (conformationally altered)						
	% homo-geneous nuclear stain	cytoplasm (intensity)	% homo-geneous nuclear stain	Number/200 cells			stippled nuclei (intensity)	cytoplasm (intensity)
				+++	++	+		
MCF10								
A1	38	-	7-8	>1	10	5	+/++	+/++
NEO	26	+/-	3-5	5	2	2	+	+
NEOT	33	+/-	12	4	15	5	+	++
TG1	35	+/-	5	2-3	4	4	++	++
TG2B	20	+	4	2	4	2	++	+/++
TG3B	32	+/-	25	7-8	14	4	++/++	+/++
TG3C	20	+/-	2	4	2	1	+++	++
TG4J	15	+	8	4	7	2	+++	++
MCF7	5	-	1-3	2	1	0	+/+++ ~	+/-

Typical cells indicating degree of intensity of homogeneous staining are marked in the photographs with 1, 2 or 3 bars.
Typical stippled nuclear staining patterns are indicated with a ~. Cells which are counted as negative or cells in the process of division (highly stained but not counted in arriving at the totals in this table) are indicated with a (0). The fields in the photomicrographs of cells stained with mAbs 1801 or 240 are not necessarily typical of the entire population of cells since they were chosen for illustration of

Abstracts presented at annual meetings:

1. Altered P53 conformation in neoplastic progression of MCF10AneoT xenograft model. P.V.M. Shekhar, J.K. Christman, M.L.-Chen, and J. Werdell. Proc. AACR, Vol. 37: 587, 1996.

2. Xenograft passage is associated with decreased frequency of N-(phosphonoacetyl)-L-aspartate resistant variants in MCF10AneoT cells. Kurumboor, S.K., Shekhar, P.V.M., and Christman, J.K. Proc. AACR, vol. 37: 32, 1996.

Manuscript Submitted:

Activation of the endogenous estrogen receptor gene in MCF10AT system cells, a potential factor in neoplastic progression of MCF10AT xenografts. P.V.M. Shekhar, M.L.-Chen, J. Werdell, G.H. Heppner, F.R. Miller, and J.K. Christman.